

Aspecific Methods of Treatment

One method for treating tumors, brachytherapy, involves injecting microscopic clumps of the protein albumin directly into the tumor. A suitable amount of radioactive phosphorous is then added through the same needle. The albumin clogs capillaries within the tumor, thereby, preventing the release of radioactive phosphorous to tissues outside the tumor. Tumor cells take up and use the phosphorous rapidly, selectively killing them with radioactivity without damaging normal cells in other parts of the body. By the time the capillaries become unclogged, all or most of the radioactive phosphorous has been absorbed by the cells comprising the tumor, leaving little to escape into adjacent tissue. This therapy, however, is difficult to implement and always carries the danger of radioactive material escaping into healthy parts of the body causing serious damage.

Robert T. Gordon in U.S. Pat. No. 4,622,952 disclosed a different method for treating tumors. This method attempts to take advantage of the observed different heat sensitivity between tumor and normal cells. It is well known that tumor cells are killed at a lower temperatures than normal cells. Thus, Gordon proposed a method using electromagnetic energy to elevate the temperature of tumor cells or tissues, to kill the tumor cells without seriously affecting normal cells.

Immunotherapy

1. Antibody Response

Many attempts have been made to kill tumor cells with polyclonal or monoclonal isoantibodies or autologous antibodies elicited against tumor-specific antigens. Generally, this method is not successful, especially when dealing with solid tumors.

2. Cytotoxic Immunity

Unfortunately, these approaches for the prevention and/or treatment of cancer have not been successful or completely satisfactory because of a number of problems, such as the absence in the vaccine of tumor antigens expressed by the tumor to be treated, poor characterization of the antigens in tumor vaccines, the contamination of vaccines by immunogenic but undesirable material, such as fetal calf serum (FCS) protein or transplantation antigens and additionally due to the antigenic heterogeneity of the cancer cells. Moreover, such tumor vaccines were often prepared from fresh tumor cells, the supply of which is limited so that the properties of the vaccines are not reproducible.

3. Current Concepts

a. Selecting an Aspecific Target Substance.

United States Patent No. 5,658,234, issued to Dunlevy in 1997 describes a method for treating a tumor comprising the steps of selecting a target substance which has at least one component with an atomic or molecular resonance frequency or frequencies different from the atomic, molecular or cellular resonant frequencies of normal cells, locating or depositing the target substance within the tumor, and irradiating the target substance with electromagnetic wave energy at a frequency or frequencies corresponding to the atomic or molecular resonance of the component such that the component absorbs energy from the electromagnetic wave, resulting in the release of heat sufficient to destroy, terminate or slow the growth of the tumor without adversely affecting the viability of normal cells.

a. Specific Melanoma Antigens
25 Melanosomal antigens can be recognized by the immune system. This has been demonstrated by immunoprecipitation of a gp75 antigen from autologous melanoma cells by serum IgG antibodies of a patient with metastatic melanoma

(Matthes, J. M., T. M. Thomson, L. J. Old, and K. O. Lloyd. (1983) A pigmentation-associated differentiation antigen of human melanoma defined by a precipitating antibody in human serum, *Int. J. Cancer.* 32:717). The gp75 antigen is a melanosomal polypeptide that is the most abundant glycoprotein synthesized by pigmented melanocytes and melanomas. (Tai, T., M. Eisinger, S. Ogata, and K. O. Lloyd. (1983) Glycoproteins as differentiation markers in human malignant melanoma and melanocytes, *Cancer Res.* 43:2773). Epidermal melanocytes, benign pigmented lesions, and primary and metastatic melanomas express gp75, but other cell types do not (Thomson, T. M., F. X. Real, S. Murakami, C. Cardo-Cardo, L. J. Old, and A. N. Houghton. (1988) Differentiation antigens of melanocytes and melanoma: Analysis of melanosome and cell surface markers of human pigmented cells with monoclonal antibodies, *J. Invest. Dermatol.* 90:459). In the present invention, it is demonstrated that gp75 cDNA had approximately 90% identity with the derived amino acid and nucleotide sequences of a mouse gene that maps to the b (brown) locus. The brown locus is a site that determines coat color and influences the type of melanin synthesized, suggesting that gp75 may regulate or influence the type of melanin synthesized.

The fact that IgG antibodies in sera of a patient with metastatic melanoma have been shown to immunoprecipitate gp75 demonstrates that immunological tolerance against gp75 can be broken. This invention therefore provides expression vectors comprising gp75 cDNA for use as a vaccine against melanoma, whereby the amino acid sequences of peptides were determined from gp75 polypeptide, which was isolated and purified by the mouse monoclonal antibody TA90, and whereby cDNA clones were isolated by screening with oligonucleotides based on the peptide sequences.

b. Human Prostatic Specific Reductase.

United States Patent No. 6,106,829, issued to He, et al. uses a human prostatic specific reductase polypeptide as a diagnostic marker for prostate cancer

and as an agent to determine if the prostate cancer has metastasized. The patent also discloses antibodies specific to the prostatic specific reductase polypeptide that may be used to target prostate cancer cells and be used as part of a prostate cancer vaccine.

5 c. Telomerase

Another method for treating tumors currently being evaluated by medical researchers makes use of a substance called telomerase, an enzyme that tumor cells produce and require to remain alive, but which normal body cells (except for sperm and hematopoietic system) neither produce nor require. This unique property of telomerase has prompted attempts to develop a drug that will block the action of the enzyme sufficiently to either inhibit the growth of new tumor cells or cause the death of older ones. Telomerase is an example of a class of substances that are often referred to as being "tumor-specific" because they are needed and/or used by tumor cells in differentially larger amounts than by normal healthy cells of the body.

Telomeres, the protein-DNA structures physically located on the ends of the eukaryotic organisms, are required for chromosome stability and are involved in chromosomal organization within the nucleus (See e.g., Zakian, *Science* 270:1601 [1993]; Blackburn and Gall, *J. Mol. Biol.*, 120:33 [1978]; Oka et al., 20 *Gene* 10:301 [1980]; and Klobutcher et al., *Proc. Natl. Acad. Sci.*, 78:3015 [1981]). Telomeres are believed to be essential in such organisms as yeasts and probably most other eukaryotes, as they allow cells to distinguish intact from broken chromosomes, protect chromosomes from degradation, and act as substrates for novel replication mechanisms. Telomeres are generally replicated in 25 a complex, cell cycle and developmentally regulated, manner by "telomerase," a telomere-specific DNA polymerase. However, telomerase-independent means for telomere maintenance have been described. In recent years, much attention has been focused on telomeres, as telomere loss has been associated with

chromosomal changes such as those that occur in cancer and aging. Importantly, telomere replication is regulated both by developmental and cell cycle factors. It has been hypothesized that aspects of telomere replication may act as signals in the cell cycle. For example, certain DNA structures of DNA-protein complex formations may act as a checkpoint to indicate that chromosomal replication has been completed (See e.g., Welling et al., *Mol. Cell. Biol.*, 13:4057 [1993]). In addition, it has been observed that in humans, telomerase activity is not detectable in most somatic tissues, although it is detected in many tumors (Welling, supra). This telomere length may serve as a mitotic clock, which serves to limit the replication potential of cells *in vivo* and/or *in vitro*. What remains needed in the art is a method to study the role of telomeres and their replication in normal as well as abnormal cells (i.e., cancerous cells). An understanding of telomerase and its function is needed in order to develop means for use of telomerase as a target for cancer therapy or anti-aging processes.

Despite the wide-ranging and expensive efforts expended in researching, developing and evaluating new treatments and cures for tumors and cancers, no truly significant advances or completely satisfactory treatments have thus far been achieved.

SUMMARY OF THE INVENTION

It is therefore an object of the present invention to provide a novel and effective treatment modality for both benign and malignant tumors. The treatment must avoid the disadvantages and dangers of the prior treatments discussed above, especially with respect to the use of radioactive substances and chemotherapy.

Another object of the present invention contemplates the ability to construct a vaccine that is universally effective against any proliferating tumor.

To achieve these objectives, a most preferred embodiment of this invention is a universal vaccine for treating tumors of any origin, having at least one

telomerase reverse transcriptase (hTRT) peptide in an amount effective for initiating and enhancing a cytotoxic T lymphocyte (CTL) response against mammalian cancer cells in a physiologically acceptable carrier. Preferably, the telomerase peptide is modified to enhance binding to a major histocompatibility complex (MHC) molecule.

The MHC molecule may advantageously be a Class I human leucocyte antigen (HLA), for example, HLA-2. Preferably, the hTRT peptide is a synthetic human telomerase reverse transcriptase peptide, but it may also be an effective synthetic homologue. Preferably, the peptide is from about 7 to about 15 amino acid residues in length, and most preferably, a 9mer. It may be effective either alone or in combination with other peptides.

The vaccine preparation described hereinabove may also comprise an adjuvant or facilitator. One highly preferred facilitator is an interleukin molecule.

Also contemplated by this invention, is a synthetic hTRT peptide advantageously restricted by a Class I major histocompatibility complex (MHC) molecule.

Another object of the invention is a method for inducing and enhancing a CTL response against cancer cells. This method comprises harvesting mammalian blood leucocytes, pulsing the leucocytes with an effective amount of hTRT, and contacting cancer cells with an effective amount of pulsed leucocytes.

This contacting may be accomplished *in vitro* or *in vivo*. The method, in its simplest form, can be used *in vitro* for determining whether a cancer patient has potential immunity against his tumor, and is a likely candidate for treatment.

Yet another object of this invention contemplates a method for targeting cytotoxic lymphocytes (CTL) to tumor cells by administering an effective amount of telomerase reverse transcriptase (TRT) peptide to a mammalian recipient, which amount is effective to attract CTL to the tumor cells. The recipient preferably is a cancer patient.

These objects and other aspects and attributes of the present invention will become increasingly clear upon reference to the following drawings and accompanying specification.

BRIEF DESCRIPTION OF THE DRAWINGS

5 **Figure 1.** Induction of CTL against hTRT in peripheral blood leucocytes (PBMC) from normal blood donors. T cells from HLA-A2+ individuals were stimulated by autologous PBMC pulsed with hTRT-derived synthetic peptides as detailed in the Material and Methods. (A). Results refer to effector cells from individual donors immunized in vitro against p540. Open circles define T2 cells 10 and closed circles T2 cells pulsed with p540 as targets. (B). Results refer to effector cells from individual donors immunized in vitro against p865. Open diamonds define T2 cells and closed diamonds T2 cells pulsed with p865 as targets. Effector to target ratios are indicated on an individual basis. Percent cytotoxicity was calculated as specified in The Materials and Methods.

15 **Figure 2.** Induction of CTL against hTRT in PBMC from prostate cancer patients. (A). Results refer to effector cells from individual patients immunized against p540. Values refer to cells tested after three rounds of in vitro stimulation. Open circles define T2 cells and closed circles T2 cells pulsed with p540 as targets. (B). Results refer to effector cells from individual patients immunized 20 against p865. Open diamonds define T2 cells and closed diamonds T2 cells pulsed with p865 as targets. Effector to target ratios are indicated on an individual basis. (C). Results refer to effector cells from individual patients immunized in vitro against p540 (circles) or p865 (diamonds). Open symbols define the HLA-A2-PC-3 prostate cancer cell line as a target. Closed symbols define the HLA-A2+ prostate cancer cell line LnCap as a target. Percent cytotoxicity was calculated as specified in the Materials and Methods section.

Figure 3. Molecular specificity of target recognition by CTL generated against hTRT peptides. (A). Cold target inhibition. ^{51}Cr -labeled LnCap cells (5×10^4 cells/ml) were mixed with T2 cells (open symbols) or T2 cells pulsed with p540 (closed circles) or p865 (closed diamond) (1 \mu g/ml) at a cold:hot target cell ratio of 5:1, 25:1 and 50:1. Patients' CTL lines 380.540.1 and 380.865.1 generated against p540 and p865, respectively, were added at an E:T ratio of 50:1. (B) Lysis of T2 cells pulsed with irrelevant HLA-A2 binding peptides. Results refer to lysis by patients' (#651) CTL generated against p540 (panel a) or p865 (panel b), and patients' (#380) CTL generated against p540 (panel c) or p865 (panel d). Closed symbols define T2 cells pulsed with p540 (circles), p865 (diamonds) and MART-1 peptide (triangles). Open circles refer to non-pulsed T2 cells. Percent cytotoxicity was calculated as specified in The Material and Methods.

Figure 4. Prostate cancer patients' CTL against hTRT are MHC Class I restricted. Patients' CTL lines 380.540.1 and 380.865.1 were tested in a ^{51}Cr -release assay 15 using as targets T2 cells pulsed with p540 (A) or p865 (B). The following inhibitory antibodies were used: murine monoclonal antibody BB7.2 (IgG2b) against MHC Class I, murine monoclonal antibody Q5/13 (IgG2a) against HLA-DR, and the engineered antibody - 1RGD3 that blocks NK cell function.

20 **Figure 5.** Human Telomerase Reverse Transcriptase (hTRT) sequence [from Nakamura et al., 1997]

Figure 6. Normal blood donor PBMC immunized in vitro against p572(Y) peptide of hTRT generate CTL that kill melanoma cells 624. The results are expressed as percent lysis and show by comparison lysis of 624 melanoma cells, and HLA-A2 + T2 target cells pulsed with the p572(Y) and p572 wild type peptides, respectively. Non specific lysis of T2 cells is shown as a control.

DESCRIPTION OF THE PREFERRED EMBODIMENT

As used herein, the terms "telomerase" and "telomerase complex" refer to functional telomerase enzymes. It is intended that the terms encompass the complex of proteins found in telomerases. For example, the terms encompass the

5 123 kDa and 43 kDa telomerase protein subunits.

Telomerase is a ribonucleoprotein enzyme which has been linked to malignant transformation in human cells. Telomerase activity is increased in the vast majority of human tumors making its gene product the first molecule common to all human tumors. The generation of endogenously-processed telomerase peptides bound to Class I major histocompatibility complex (MHC) molecules could therefore target cytotoxic T lymphocytes (CTL) to tumors of different origins. This could advance vaccine therapy against cancer provided that precursor CTL recognizing telomerase peptides in normal adults and cancer patients can be expanded through immunization. Applicant demonstrates here that the majority of normal individuals and patients with prostate cancer

10 immunized in vitro against two HLA-A2.1 restricted peptides from telomerase reverse transcriptase (hTRT), develop hTRT specific CTL. This suggests the existence of precursor CTL for hTRT in the repertoire of normal individuals and in cancer patients. Most importantly, cancer patients' CTL specifically lysed a variety of HLA-A2+ cancer cell lines, demonstrating immunological recognition of endogenously-processed hTRT peptides. Moreover, *in vivo* immunization of HLA-A2.1 transgenic mice generated a specific CTL response against both hTRT peptides. Based on the induction of CTL responses *in vitro* and *in vivo*, and the susceptibility to lysis of tumor cells of various origins by hTRT CTL, Applicant

15 suggests that hTRT could serve as a universal cancer vaccine for humans.

INTRODUCTION

Telomerase is a unique ribonucleoprotein that mediates RNA-dependent synthesis of telomeric DNA (1), the distal ends of eukaryotic chromosomes that

stabilize the chromosomes during replication (2, 3). When activated, telomerase synthesizes telomeric DNA and compensates for its loss with each cell division (4). Since telomeres shorten progressively with successive cell divisions, telomere length is considered to mirror the replicative history of cell lineage (5) and cell population dynamics (6, 7). In mice, telomerase appears to play an essential role in the long-term viability of high-renewal organ systems such as the reproductive and haemopoietic systems (8).

Maintenance of a constant telomere length ensures chromosomal stability, prevents cells from aging, and confers immortality (9-11). Mice lacking telomerase RNA show that telomerase activation is a key event in malignant cell transformation (8, 12, 13). In humans, *in vitro* studies show that the long-term ectopic expression of telomerase reverse transcriptase (hTRT) in normal fibroblasts is sufficient for immortalization but not malignant transformation (14). However, the expression of hTRT in combination with two oncogenes (SV40 T antigen and Ras) promotes tumor transformation in normal human epithelial and fibroblast cell lines (15). These transformed cells form tumors in nude mice. Thus, although telomerase per se is not tumorigenic, it plays a direct role in oncogenesis by allowing pre-cancerous cells to proliferate continuously and become immortal. The PCR-based TRAP assay (16) reveals a striking correlation (>80%) between high telomerase activity and tumors of different histological origins and types (17, 18). In contrast, normal tissues display little or no telomerase activity (18, 19). Therefore, telomerase expression in tumors is much greater than HER2/neu and mutated p53, which range between 30% and 50% respectively (20, 21). From the foregoing, it is reasonable that expression of hTRT in cancer cells is a likely source of peptides that, upon association with major histocompatibility complex (MHC) Class I molecules, could target cytotoxic T lymphocytes (CTL) to cancer cells. An interesting analogy exists with HIV-1 reverse transcriptase, an enzyme similar to hTRT, which gives origin to peptide/MHC Class I complexes that target CTL responses to virus infected

cells (22). Thus, since high telomerase activity is widespread among human tumors, hTRT could serve as a universal tumor antigen for immunotherapy and vaccine approaches.

hTRT is encoded in the genome and is in all respects a self antigen.

5 Consequently, CD8+ T lymphocytes with a receptor for MHC/hTRT peptide complexes are expected to be eliminated during thymic negative selection, reducing the potential precursor T cell repertoire and imposing limitations on their expansion upon encounter with tumor cells in adult life. Additionally, stimulation by antigen in the absence of a second signal induces clonal anergy (23), further hampering the potential repertoire. The extent to which these events affect the normal adult repertoire, and whether or not exposure to hTRT during cancer formation has any adverse effect on the ability of cancer patients to respond, is not known. Because answering these questions is relevant to future strategies of immune intervention targeted at hTRT, the ability of normal individuals and cancer patients to mount a CTL response *in vitro* against two hTRT peptides restricted by the HLA-A2 allele was analyzed.

MATERIALS AND METHODS

Example 1

Synthetic peptides

20 hTRT synthetic peptides p540 (540ILAKFLHWL548), p865 (865RLVDDFLLV873) and MART-1 (27AAGIGLTV33) were purchased from the Biopolymer Synthesis Center (CalTech, Pasadena, CA). Synthetic peptides 128TPPAYRPPNAPL140 of the hepatitis B core antigen (HBc), S71YLSGANIQL579 of carcinoembryonic antigen (CEA),

25 476VLYRYGSEFSV486 of melanoma antigen Bp100, 476ILKEPVHGV484 of HTV-1 reverse transcriptase were purchased from Neosystem (Strasbourg, France).

Human blood cells

Buffy coats from normal donors were purchased from the San Diego Blood Bank. HLA-A2 + individuals were selected by FACS screening using monoclonal antibody BB7.2. Prostate cancer patients were recruited through the

5 Division of Urology (University of California, San Diego). Blood from these patients was obtained by venipuncture. HLA-A2 + individuals were selected by FACS screening using monoclonal antibody BB7.2. Blood collection and experiments were performed in accordance with an approved IRB.

Tumor cell lines

10 T2 cells were a kind gift of Dr. Peter Creswell (Yale University). Melanoma cell lines 624 and 1351 were the kind gift of Dr. John Wunderlich (National Cancer Institute, Bethesda, MD). Prostate cancer cell lines LnCap and PC-3 were the kind gift from Dr. Antonella Vitiello (PRI Johnson, La Jolla CA). Breast, colon and lung tumor cell lines were obtained from ATCC, Rockville, MD.

Example 2

In vitro immunization

PBMC were separated by centrifugation on Ficoll-Hyphaque gradients and plated in 24-well plates at 5x105 cells/ml/well in RPMI-1640 supplemented with 10% human AB+ serum, L-glutamine and antibiotics (CM). Autologous PBMC (stimulators) were pulsed with hTRT synthetic peptides p540 or p865 (10 µg/ml) for 3 hours at 37 °C. Cells were then irradiated at 5000 rads, washed once, and added to the responder cells at a responder/stimulator ratio ranging between 1:1 and 1:4. The next day, 12 IU/ml IL-2 (Chiron Co., Emeryville, CA) and 30 IU/ml 25 IL-7 (R&D Systems, Minneapolis, MN) were added to the cultures. Lymphocytes were re-stimulated weekly with peptide-pulsed autologous adherent cells as

follows. First, autologous PBMC were incubated with hTRT peptide (10 µg/ml) for 3 hours at 37 °C. Non-adherent cells were then removed by a gentle wash and the adherent cells were incubated with fresh medium containing the hTRT peptide (10 µg/ml) for an additional 3 hours at 37 °C. Second, responder cells from a previous stimulation cycle were harvested, washed and added to the peptide-pulsed adherent cells at a concentration of 5x105 cells/ml (2 ml/well) in medium without peptide. Recombinant IL-2 and IL-7 were added to the cultures on the next day.

Example 3

In vivo immunization

HHD mice were immunized subcutaneously at the base of the tail with 100 µg of individual hTRT peptide emulsified in incomplete Freunds' adjuvant (IFA). Half of the mice were immunized with the hTRT peptide and 140 µg of the helper Peptide TPPA YRPPNAPII, which corresponds to residues 128-140 of the 15 hepatitis B core antigen (HBc) (25).

Example 4**HLA-A2.1 binding/stabilization assay**

The relative avidity was measured as previously described (25). Briefly, T2 cells were incubated overnight at 37 °C in RPMI supplemented with human β2-microglobulin (100 ng/ml) (Sigma, St. Louis, MO) in the absence (negative control) or presence of the test peptide or the reference peptide 476ILKEPVHGV484 of HIV-1 reverse transcriptase at various final peptide concentrations (0.1-100 µM). Cells were incubated with Brefeldin A (0.5 µg/ml) for one hour and subsequently stained with a saturating concentration of 20 monoclonal antibody BB7.2 for 30 minutes at +4 °C followed by washing and a second incubation with a goat antibody to mouse Ig (Fab)2 conjugated to FITC 25

(Caltag, South San Francisco). Cells were then washed, fixed with 1% paraformaldehyde and analyzed in a FACS Calibur cytofluorimeter (Becton&Dickinson, San Jose, CA). The mean fluorescence intensity of each concentration minus that of cells without peptide was used as an estimate of peptide binding. Results are expressed as values of RA, which is the ratio of the concentration of test peptide necessary to reach 20% of the maximal binding by the reference peptide over that of the reference peptide so that the lower the value the stronger the binding. Dissociation of the test peptide from the HLA-A2.1 molecule reflects the half-life of fluorescence intensity of the peptide/MHC complex over time. The half-life of the complex (DC50) refers to the time (hours) required for a 50% reduction of the T0 mean fluorescence intensity (25).

Synthetic peptides 571YLSGANLNL579 of carcinoembryonic antigen (CEA) and 476VLYRYGSFSV486 of melanoma antigen gp100 were used as internal controls to account for inter-tests variability and for consistency with previously 15 reported RA and DC50 measures (25).

Example 5**Cytotoxicity assay**

(a) The induction of CTL in human PBMC was monitored in a conventional 51Cr-release assay. Briefly, peptide-pulsed TAP-HLA-A2.1+ 20 human T2 cells were incubated with 10 µg of hTRT peptides or with the MART-1 control peptide for 90 minutes during labeling with 51Cr. After washing, the target cells were added to serially diluted effectors in 96-well microplates. After a 6 hours incubation at 37 °C, supernatants were harvested and counted in a Trilux Betaplate counter (Wallac, Turku, Finland). Results are expressed as the 25 percentage (%) of specific lysis and determined as follows: [(experimental cpm - spontaneous cpm)/(maximum cpm - spontaneous cpm)] x 100. (b) The induction of CTL in HHD mice was assessed as follows. Spleen cells were harvested 7 days after immunization and were restimulated *in vitro* with the corresponding hTRT

peptide and LPS (25 μ g/ml)-stimulated irradiated (5000 rads) syngeneic spleen cells. After six days of culture the cells were harvested and tested for their ability to lyse HHD-transfected/TAP- RMA-S cells in a 4 hour 51Cr-release assay (25). Specific lysis was calculated as indicated in the legend of Figure 1. Values refer to maximal cytotoxicity measured for individual responder mice at an effector to target ratio of 60:1.

RESULTS

Example 6

Identification and Analysis of HLA-A2.1-restricted hTERT Peptides

The amino acid sequence of hTERT (locus AF015950) (19) was analyzed for 9mer peptide sequences containing known binding motifs for the HLA-A2.1 molecule [52; 35; 60], a subtype encompassing 95% of HLA-A2 allele which is expressed in about 50% of the Caucasian population (26-28). Peptides were identified by reverse genetics based on canonical anchor residues for HLA-A2.1 (29), and by using the software of the Bioinformatics & Molecular Analysis Section (NIH) available at http://bimas.dcr.nih.gov/molbio/hla_bind/index.html which ranks 9mer peptides on a predicted half-time dissociation coefficient from HLA Class I molecules (30). From an initial panel of ~30 candidate peptides Applicant retained two sequences, 540ILAKFLHWL548 and 865RLVDDFLLV873, denoted hereunder as p540 and p865.

Since the immunogenicity of MHC Class I-restricted peptides reflects to some degree their binding and stabilizing capacity for MHC Class I molecules (31-33) Applicant sought direct proof of the strength of interaction between the two hTERT peptides and the HLA-A2.1 molecule in a conventional binding/stabilization assay that uses the antigen-transporting deficient (TAP-) HLA-A2.1+ human T2 cells. The relative avidity (RA) calculated in reference to 476LKEPVHGV484 of HTV-1 reverse transcriptase, a canonical high binder peptide (25), was 2.9 and 2.5 for

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p540 and p865, respectively (Table 1). The stability of each peptide bound to HLA-A2.1 was measured as the half-life of the complex (DC50) and was in the order of 4-6 hours for p540 and 2-4 hours for p865, respectively. Collectively, these measurements indicate that both hTERT peptides are excellent binders to HLA-A2.1 albeit p865 has a faster dissociation rate.

Example 7

CTL Response Against hTERT in Normal Human Individuals

The presence of precursor T cells for both hTERT peptides and their expansion upon antigen stimulation were tested using peripheral blood lymphocytes (PBMC) of 10 HLA-A2+ normal blood donors in an in vitro immunization assay. Nine out of 10 individuals responded to immunization generating T cells that lysed peptide-pulsed T2 cells as targets starting from the third round of peptide stimulation. All nine responders generated CTL specific for p540 and seven responded against p865 (Figure 1A and B). The values of maximal lysis varied from individual to individual and ranged between 28-68% and 20-68%, respectively. In two instances (donor 975 and 980) there was a lower but measurable non-specific lysis, possibly due to contaminant NK cells. Thus, by random testing of normal HLA-A2+ individuals, it was clearly established that both hTERT peptides are immunogenic, implying that precursor CTL for hTERT are present in the peripheral adult repertoire.

Example 8

CTL Response Against hTERT in Cancer Patients

Whether or not CTL against hTERT could also be induced in cancer patients was studied in four HLA-A2.1+ individuals with clinical and histological diagnosis of prostate cancer. All four patients were refractory to hormonal therapy, three had metastases and none had prostatectomy. In prostate cancer, the

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most common cause of cancer in men, high hTRT expression has been documented in 84% of cases (34). Marked lysis of peptide-pulsed T2 cells was observed in 3 out of 4 individuals after three rounds of in vitro stimulation (Figure 2,A and B). Both peptides yielded comparable CTL responses in all three individuals with maximal lysis ranging between 27-49% and 48-52%, respectively. CTL against both peptides lysed LnCap, a HLA-A2.1+ prostate cancer cell line, with maximal lysis ranging between 24-36% for p540 and 12-40% for p865. Prostate cancer cell line PC-3, which is HLA-A2.1-, was used as control and was not lysed (Figure 2,C). Both prostate cancer cell lines tested positive for hTRT by the TRAPez (telomerase detection assay, INTERGEN) (not shown), suggesting that the CTL generated against the synthetic peptides might lyse cancer cells by recognizing hTRT peptide/MHC Class I complex at the surface of cancer cells.

Cold target competition experiments were performed in an attempt to understand if lysis of the LnCap tumor cell line was specific for endogenously-processed hTRT peptides. In these experiments the lysis of LnCap cells by CTL from a prostate cancer patient was competed for by T2 cells pulsed in vitro with p540 or p865 (10 μ g/ml). Peptide-loaded T2 cells caused a dose-dependent inhibition of lysis of LnCap cells in both peptide combinations (Figure 3,A). Applicant further assessed the specificity of the CTL generated against each one of the two hTRT peptides by testing them on T2 targets pulsed with irrelevant HLA-A2 binding peptides. Neither T2 cells pulsed with peptide 27AAGIGLTV35 from the melanoma antigen MART-1 nor T2 cells pulsed with a non-homologous hTRT peptide were lysed (Figure 3,B). Collectively, these studies show that 1) patients' CTL are specific for the hTRT peptide used to induce them, and 2) lysis of prostate cancer cells is mediated by, and is specific for, endogenously-processed hTRT peptides complexed with HLA-A2.1 molecules, suggesting chemical identity between naturally processed peptides on

tumor cells and the synthetic peptides used for immunization. Formal validation will require elution of peptides from tumor cells and their analysis by tandem mass spectrometry (35). Studies on MHC restriction were performed using blocking antibodies. Lysis of peptide-pulsed T2 cells by CTL lines generated from a prostate cancer patient was inhibited by the anti-MHC Class I monoclonal antibody BB7.2 in both peptide combinations (Figure 3), but not by the anti-MHC Class II monoclonal antibody Q5/13 (36) nor by transfectoma antibody _1RGD3 that blocks NK cells (37). By two-color FACS analysis, the phenotype of T cells proliferating after three rounds of in vitro stimulation with hTRT peptide was CD3+ (78%), CD8+ (37%), CD4+ (36%) and CD16/56 (6%). Collectively, these experiments confirm that effector T cells generated by in vitro immunization are MHC Class I-restricted (CD8+) T cells which do not possess NK activity. hTRT is expressed in normal cells such as circulating B and T cells, germinal center B cells, thyocytes and CD34+ progenitor hematopoietic cells (6, 7, 38). This implies that CTL generated against hTRT peptides could engender an autoimmune attack on normal cells. To this end, Applicant verified whether cancer patients' CTL would lyse HLA-A2+ CD34+ cells. Neither CTL against p540 nor those against p865 induced any lysis over a wide range of effector to target (E:T) ratios (not shown). Thus, at least with respect to hematopoietic stem cells an autoimmune attack appears unlikely. This is consistent with the fact that activated T cells were not lysed by hTRT CTL in culture.

Example 10

CTL Response Against hTRT in HLA-A2.1-Transgenic Mice

Whether peptides can serve as immunogens in vivo and elicit a CTL response depends on a variety of factors such as the mode of immunization, suitable activation of antigen presenting cells, the frequency of precursor cells, and binding and stabilization of MHC Class I molecules by peptide. In this study Applicant demonstrated (Table I) that both peptides bind to HLA-A2.1 with a RA

>3 but possess different dissociation rates. In either case Applicant was able to generate CTL responses *in vitro* from PBMC of normal blood donors as well as prostate cancer patients. Therefore, a reasonable expectation would be that they may also be immunogenic *in vivo*. To test this possibility Applicant immunized H-2Db^{-/-}, β 2m^{-/-}, HLA-A2.1+ monochain transgenic HHD mice (39). In these mice the peripheral CD8+ T cell repertoire is essentially educated on the transgenic human molecule. Therefore, HHD mice are an excellent tool to assess at the pre-clinical level the ability of individual peptides to induce HLA-A2.1 restricted CTL responses *in vivo* (25).

Both p540 and p865 were able to induce specific CTL responses (Table II) although differences were noted. In fact, p540 induced CTL whether alone or in combination with a helper peptide (66 vs. 80 % responders). In contrast, a high (70%) response against p865 was obtained only when its immunogenicity was increased by association with the helper peptide. The different immunogenicity of the two hTTR peptides was also reflected by the magnitude of individual responses (55.8/9.4 vs. 20/11.5 % lysis) against p540 and p865 with helper peptide, respectively. This is consistent with the observation that two normal blood donors responded to immunization against p540 but failed to respond against p865 (Figure 1). Thus, there is an overall correlation between the results of binding/stabilization of the HLA-A2.1 molecule, the results of immunogenicity *in vitro* of human PBMC, and the response *in vivo* in HHD mice. Finally, to exclude the development of untoward autoimmunity HHD mice immunized against hTTR peptides were monitored with respect to the number of circulating B lymphocytes. Using a dual stain (B220 and anti-Ig) FACS analysis Applicant found no decrease in circulating B cells in immunized mice when compared to normal HHD mice (not shown). Furthermore, no enlarged mesenteric lymph nodes nor cellular infiltrates in the liver were noticed after immunization (not shown).

Example 11

Cancer Patients' CTL Kill Tumor Cells of Various Origins and Types

Because CTL generated against p540 and p865 recognize naturally-processed hTTR peptides on LnCap prostate cancer cells and hTTR activity is expressed at high levels in the vast majority of human cancers, recognition of hTTR-derived peptides by CTL could mediate killing of a wide variety of cancer types. CTL lines from a prostate cancer patient were used in a 51Cr-release assay to assess lysis of HLA-A2+ tumor cell lines of breast, colon, lung, and melanoma origin as targets. By the TRAPeze assay all these cell lines were hTTR positive.

10 Peptide-pulsed T2 cells and LnCap prostate cancer cell line served as positive controls (Table III). All cell lines but the SW480 colon cell line were lysed by CTL generated against p540 (range lysis 39-48 %). On the other hand, all cell lines but the H99 lung cell line were lysed by CTL generated against p865 (range lysis 37-41 %). The cytotoxic activity was dependent on expression of the HLA-A2 molecule since tumor-matched cell lines of a different HLA type were not lysed. Collectively, these data indicate that hTTR peptides such as p540 and p865 are naturally-processed in a variety of tumor cell types.

15 The antigen-recognition activity of T cells is intimately linked with recognition of MHC (HLA in humans) molecules. This complex is located on chromosome 6, and encompasses nearly 200 genes encoding for MHC class I and class II among others. The initial discovery is in relation to the HLA-A2 allele which is expressed in about 50% of the Caucasian population (56). About 95% of HLA-A2+ white individuals express the HLA-A2.1 subtype (53).

20 The majority of peptides bound to MHC class I molecules have a restricted size of 9±1 amino acids and require free N- and C-terminal ends (52; S9; 61). In addition to a specific size, different class I molecules appear to require a specific combination of usually two main anchor residues within their peptide ligands (52; 59). In the case of the human allele HLA-A2.1, these anchor residues have been

described as leucine (L) at position 2 and L or valine (V) at the C-terminal end (52). More recently, Ruppert et al. found that a "canonical" A2.1 motif could be defined as L or M (methionine) at position 2 and L, V, or I (isoleucine) at position 9 (60).

5 Additional criteria were used to refine the selection process. Each of the non-anchor residues (position 1,3,4,5,6,7,8) has significant effect of the A2.1 binding (60). More specifically, some amino acids at position 1, 3, 6, 7, and 8 virtually abolish A2.1 binding capacity of peptides (60). Therefore, Applicant excluded all peptides with the following amino acids at the position specified: D (aspartate) and P (proline) at position 1; K (lysine) at position 3; R (arginine) or G (glycine) at position 6; and E (glutamate) at position 7 or 8. Through this selection Applicant excluded 12 and retained 27 peptides. By taking into account the frequency of each amino acid in each of the non-anchor positions for many 9mer peptides (60) Applicant defined a more accurate A2.1 binders and 10 out of the 15 peptides (Table IV):

described on a predicted half-time dissociation from HLA class I molecules deduced from (58). In our pilot studies one of the peptides identified using the "manual" approach - P865 - ranked among the top 5 HLA-A2-binding peptides identified through the software-guided analysis. Another peptide - P540 - ranked 9 (60).

5 at the top in the software-guided analysis

Applicant used two such peptides 540ILAKFLHWL549 and 865RLVDDFLLV873, denoted as p540 and p865. Both peptides are able to induce a CTL response *in vitro* in normal blood donors and in patients with prostate cancer. Applicant has demonstrated that the same peptides are also able 10 to induce a CTL response *in vitro* in patients with melanoma. A synopsis of these 15 studies is shown in Table V.

Collectively, it appears that p540 induced a CTL response in 3 out of 4 HLA-A2+ patients. P865 induced a response in two patients only. It should be noted that patient 00 was concomitantly being immunized with dendritic cells + melanoma peptides (peptides other than hTRT peptides) and had a high 15 background making it difficult to decided whether a specific response to hTRT had been induced.

Table V
INDUCTION OF CTL IN VITRO IN PATIENTS WITH MELANOMA

Patient Code	CTL to p540	CTL to p865	Comments
28-7	50%	14%	
00	<5%	<5%	Concomitantly immunized with DC; High background
25	66-5	49%*	1% *Measurable NK activity (17%)
	22-1	40%	43%

The peptide selection was confirmed using the application available online at the web site of the Bioinformatics&Molecular Analysis Section of NIH (http://bimms.dcr.nih.gov/molbio/ha_bind/index.html) that ranks potential 9mer

Additional new findings came from exploring the immunogenicity of other hTERT peptides. In particular, three peptides were tested whose sequence in the native hTERT molecules is shown below in Table VI:

Unlike p540, which was characterized as having a high affinity binding (slow half time dissociation) to HLA-A2 (Table V), these peptides have an estimated half time dissociation score faster than prototype p540. Calculations were made using the program (http://bimbas.dor.nih.gov/hla_bind/index.html).

Table VI
ADDITIONAL SEQUENCE OF WILD TYPE AND MODIFIED hTERT PEPTIDES

Name of Peptide	Wild Type Sequence	Modified Sequence
p152	1 ⁵² LLARCALFV ⁶⁰	1 ⁵² YLLARCALFV ⁶⁰
p555	5 ⁵⁵ ELLRSFFYV ⁶³	5 ⁵⁵ YELLRSFFYV ⁶³
p572	7 ⁵² LFFYRKSV ⁶⁰	5 ⁷² YLFYRKSV ⁶⁰

Applicant then proceeded at a single residue (Y) modification in position 1 which is supposed to increase the binding affinity to HLA-A2 and also its immunogenicity (60). The new modified sequences are shown in Table VI.

PBMC from three normal HLA-A2+ individuals were immunized with the Y-modified peptides. The results are summarized as follows (Table VII):

CTL generated against p572 were also able to lyze the hTERT+ /HLA-A2+ melanoma cell line 624. The dose response curve of killing of melanoma 624 is shown in Figure 6. The antigen-recognition activity of T cells is intimately linked with recognition of MHC (HLA in humans) molecules.

DISCUSSION

Applicant has demonstrated that hTERT peptides can expand precursor CTL in PBMC of normal individuals and patients with prostate cancer, and induce in both

instances MHC Class I-restricted, peptide-specific CTL responses. Therefore, the first major implication from these findings is that the available CTL repertoire for hTERT is similarly preserved not only in normal individuals as recently reported (24) but also, and more importantly, in individuals with cancer. This suggests that exposure to cancer does not cause deletion or anergy of clonotypes specific for hTERT. Since the three patients responding to immunization were resistant to hormone therapy and had metastases, it was surprising that hTERT CTL could be induced at such an advanced stage of disease generally characterized by immunosuppression. Based on these considerations, one could predict that since the frequency of precursors from

Table VII

The immunogenic capability of "Y" modified hTERT peptides.

Donor	Immunogen	CTL Against Target Cells pulsed With . . .		
		p152(Y)	p555(Y)	p572(Y)
15	218	p152(Y)	7%	
		p555(Y)		
20	46%	p572(Y)		
			4%	
219	219	p152(Y)	2%	
		p555(Y)		8%
25		p572(Y)		
				4%
272	272	p152(Y)	1%	
		p555(Y)		1%
30		p572(Y)		
			27%	5%

PMIC is high enough to permit their expansion in vitro and because hTERT peptides bind to MHC Class I with sufficient avidity, the two peptides identified in this study may be used for vaccination of HLA-A2+ cancer patients.

The finding that prostate cancer patients' CTL mediate efficient lysis of a variety of HLA-A2+ cancer cells such as prostate, breast, colon, lung and melanoma is

unprecedented. Based on the values of specific lysis it appears as if these cancer cells are equally effective in processing and presenting the same endogenous hTRT peptides. Therefore, a second major implication of our study is that similar hTRT peptides are expressed and complexed with MHC Class I molecules on a variety of cancer cells of different histological origins and types. This renders them susceptible to destruction by CTL and underscores the potential advantage hTRT immunization may have in the control of primary tumors and metastases in a large variety of cancer types in humans.

The future of hTRT-based vaccination will also depend on the type of side effects that may follow immunization. Since hTRT is expressed in stem cells and mature hemopoietic cells (6, 7, 38), the possibility exists that hTRT vaccination could result in autoimmunity and destruction of normal cells. In our hands cancer patients' CTL specific for either p540 or p865 failed to lyse HLA-A2+ CD34+ cells. Similarly, CTL against p540 raised in normal individuals did not lyse HLA-A2+ CD34+ cells (24). Together with the lack of overt autoimmune defects in hemopoietic cells and in the liver in HHD mice following vaccination with hTRT peptides, Applicant provisionally concludes that CTL specific for hTRT are unlikely to trigger autoimmunity against normal cells. Possibly, the quantity of hTRT peptides generated under physiological lineage/clonotype activation and differentiation is insufficient to mediate lysis by CTL. Whether the same holds true for germ cells of reproductive organs for which little is known about CD8 T cell-mediated autoimmunity, can only be speculated. While additional experiments are needed, the fact that autoimmunity does not develop after immunization against tumor antigens shared by normal tissues (48, 49), including the lymphoid tissue (50) and reproductive organs (51), supports the view that hTRT-based vaccination in cancer patients may be possible and safe.

Methods to implement such hTRT-based vaccination will include the variety of methods currently in use, such as synthetic peptides, synthetic peptides in immunological adjuvant, dendritic cells pulsed with synthetic peptides, naked DNA and RNA. In addition, Applicant anticipates that effective vaccination can be achieved using transgenic cells. For instance, genes under a specific lymphocytic promoter can be engineered to code for desired hTRT peptides, transfected and expressed in lymphocytes from an individual (e.g., a cancer patient), and the patient's own lymphocytes can be used

for vaccination, since lymphocytes process and present peptides to T lymphocytes, hence effecting the of vaccination.

In conclusion, based on the demonstration that precursor CTL specific for two hTRT peptides can be expanded in patients with cancer, their CTL recognize the same 5 hTRT peptides on tumor cells of various origins and histological types, and a strong in vivo CTL response against both hTRT peptides was induced in HLA-A2.1+ monochain transgenic mice, Applicant suggests that hTRT can be regarded as a universal cancer antigen and its peptides as the substrate for a possible universal cancer vaccine for humans.

10 In accordance with the preceding explanation, variations and adaptations of the vaccine and methodology of the present invention will suggest themselves to a skilled practitioner in the medical arts. In the spirit of this invention, these and other possible variations and adaptations of the present invention, and the scope of the invention, should be determined in accordance with the following claims,

15 only, and not solely in accordance with that embodiment within which the invention has been taught.

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CLAIMS

What is claimed is:

1. A universal vaccine for treating tumors of any origin, comprising:
5 at least one telomerase reverse transcriptase(hTRT) peptide in an amount effective for initiating and enhancing a cytotoxic T lymphocyte (CTL) response against mammalian cancer cells; and a physiologically acceptable carrier.
2. The vaccine according to claim 1, wherein the telomerase peptide is modified to enhance binding to a major histocompatibility complex (MHC) molecule.
3. The vaccine according to claim 2, wherein the MHC molecule is a Class I.
4. The vaccine according to claim 3, wherein the MHC molecule is a human leucocyte antigen (HLA).
5. The vaccine according to claim 4, wherein the MHC molecule is HLA-2.
6. The vaccine according to claim 1, wherein the hTRT peptide is a human telomerase reverse transcriptase peptide.
7. The vaccine according to claim 6, wherein the peptide is from about 7 to about 15 amino acid residues in length.
8. The vaccine according to claim 1, wherein the peptide is effective alone.
9. The vaccine according to claim 1, wherein the peptide is effective in combination with other peptides.
10. The vaccine according to claim 1, wherein the vaccine also comprises an adjuvant.

11. The vaccine according to claim 1, wherein the carrier is a mammalian cell.

12. The vaccine according to claim 11, wherein the carrier mammalian cell is a transfected or transgenic cell.

13. A synthetic hTRT peptide restricted by a Class I major histocompatibility complex (MHC) molecule.

14. A method for inducing and enhancing a CTL response against cancer cells, comprising:
10 harvesting mammalian blood leucocytes; pulsing with an effective amount of hTRT; and contacting cancer cells with an effective amount of pulsed leucocytes.

15. The method according to claim 13, wherein the contacting is accomplished *in vitro*.

16. The method according to claim 13, wherein the contacting is accomplished *in vivo*.

15 17. A method for targeting cytotoxic lymphocytes (CTL) to tumor cells by administering an effective amount of telomerase transcriptase (TRT) peptide to a mammalian recipient, which amount is effective to attract CTL to the tumor cells.

18. The method according to claim 16, wherein the recipient is a cancer patient.

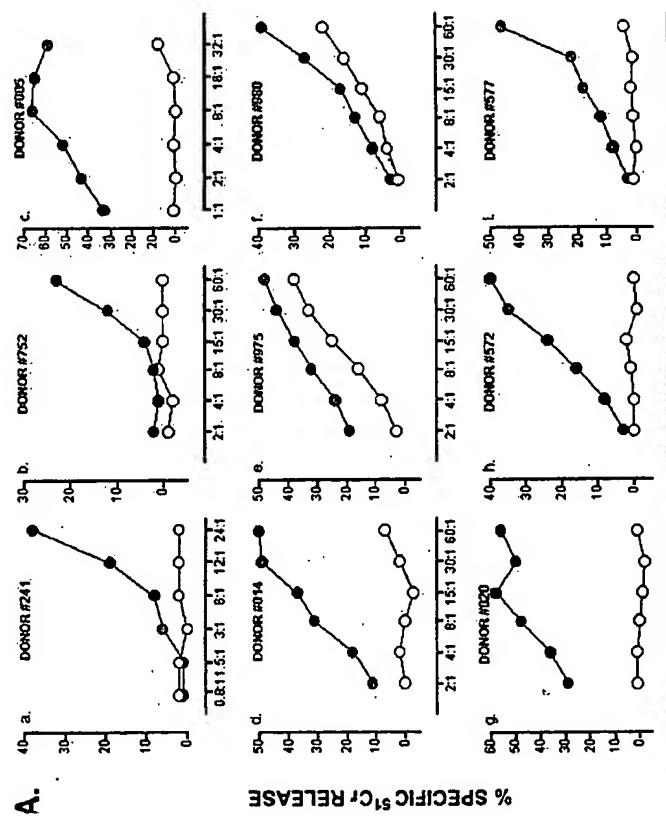


Figure 1 A



Figure 1 B

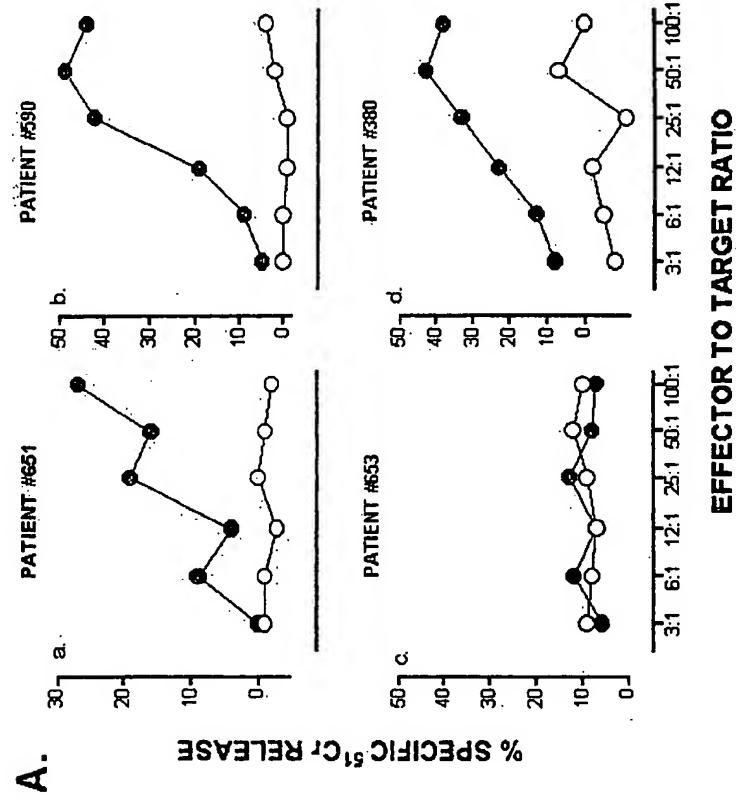


Figure 2 A

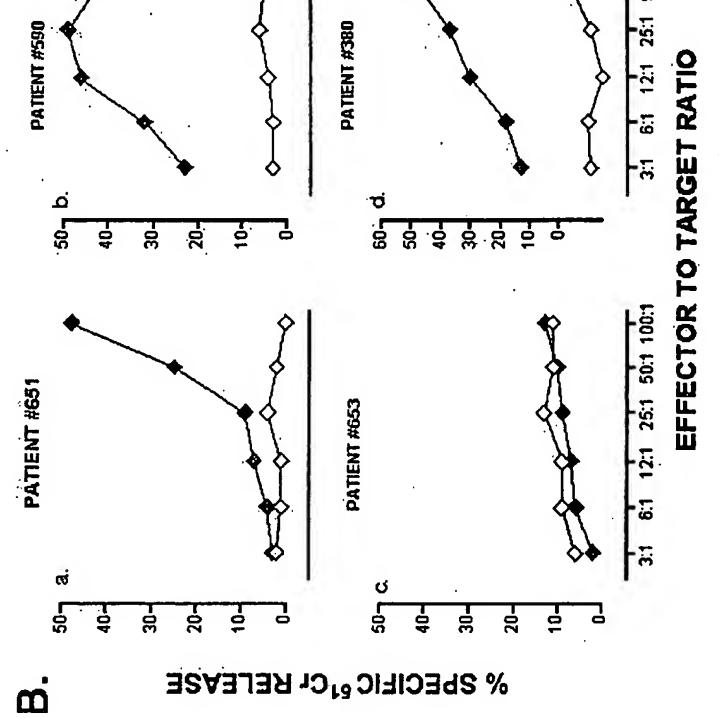


Figure 2 B

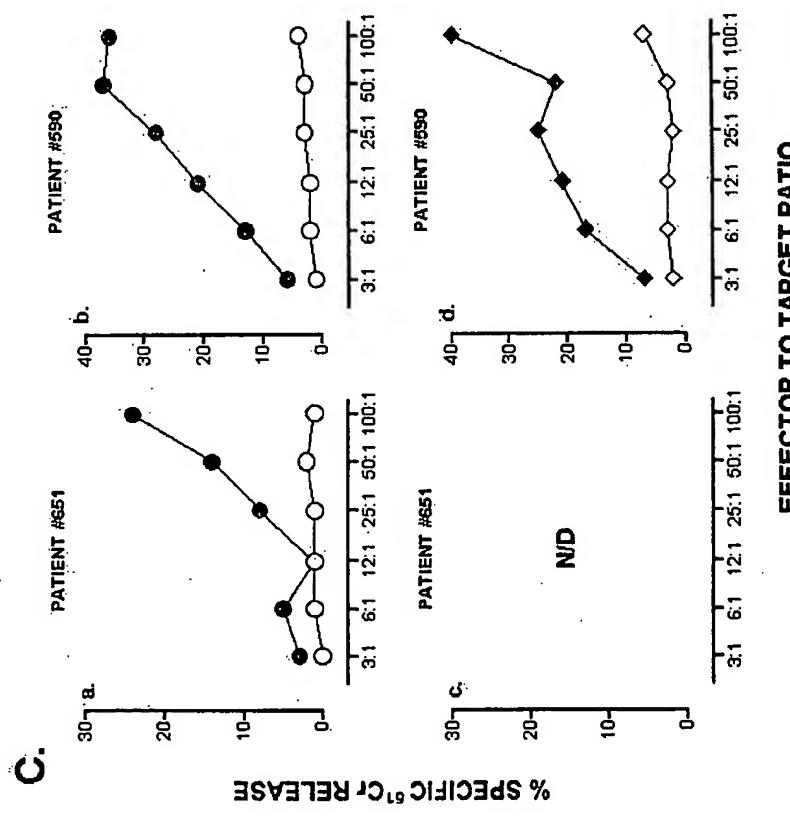


Figure 2 C

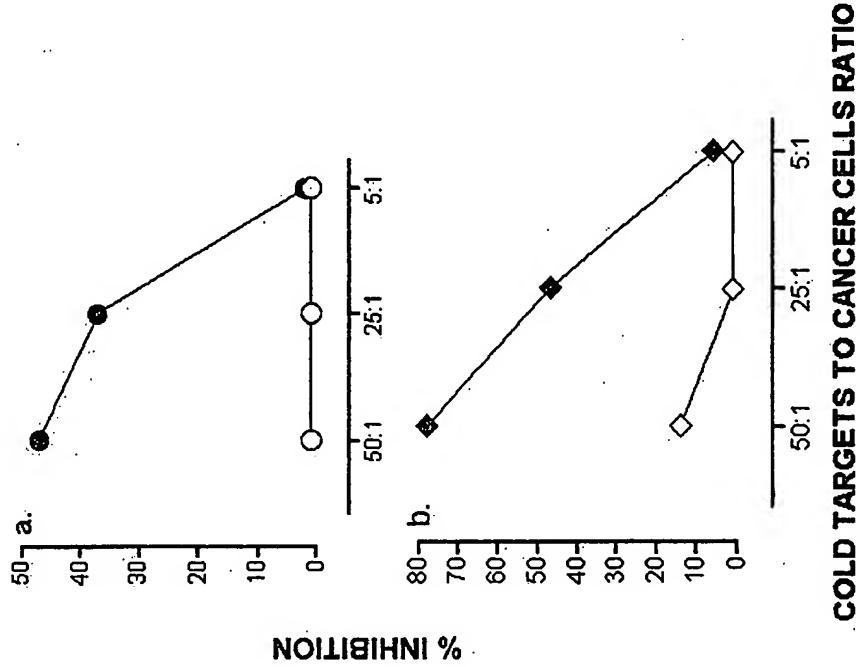


Figure 3 A

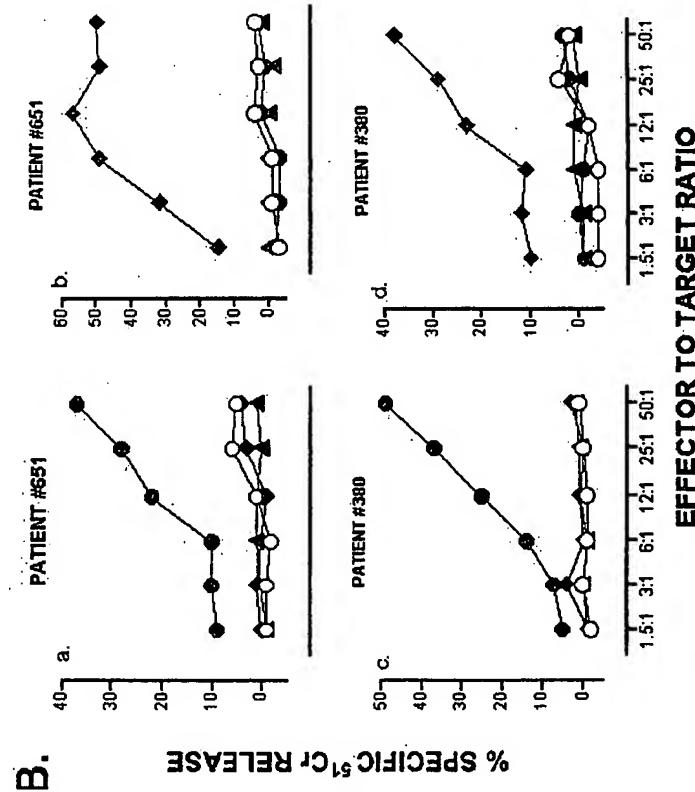


Figure 3 B

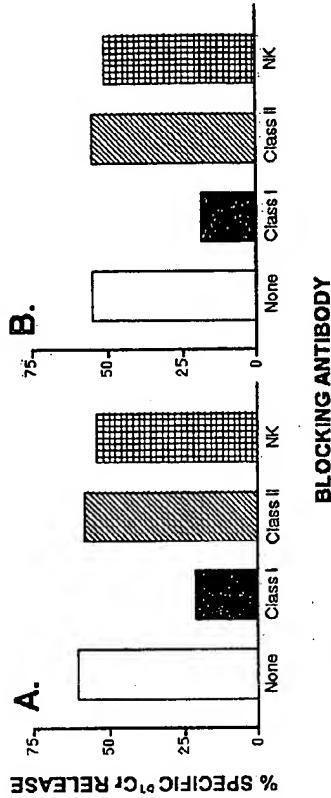


Figure 4

"M" at position 2, "V, L or I" at position 9
"L" at position 2, "V" at position 9

Figure 5

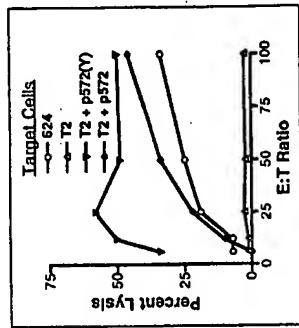
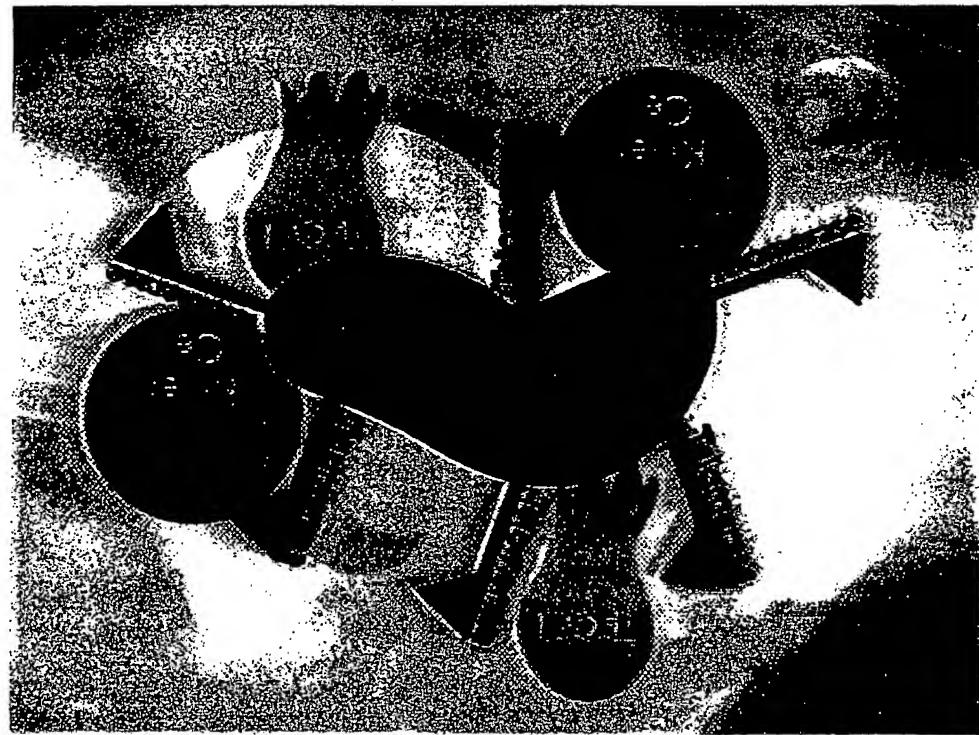


Figure 6



SEQUENCE

1: NP_003210 telomerase Hlink. Pubmed. Related Sequences.
 Nucleotide, OMIM, LinkOut

5 Taxonomy, OMIM, LinkOut
 reverse transcriptase;
 hEST2 [Homo sapiens]

10 LOCUS NP_003210 1132 aa FRI 31-OCT-2000
 DEFINITION telomerase reverse transcriptase; hEST2 [Homo sapiens].

15 ACCESSION NP_003210
 PID 94507439
 VERSION NP_003210.1 GI:4507439
 DBSOURCE accession NM_003219.1

KEYWORDS

19 SOURCE human.

20 ORGANISM Homo sapiens
 Eukaryota; Metazoa; Chordata; Craniata; Vertebrata;
 Euteleostomi; Mammalia; Eutheria; Primates; Catarrhini; Hominoidea; Homo.

REFERENCE 1 (residues 1 to 1132)

25 AUTHORS Nakamura T.M., Marin G.B., Chapman K.B., Weinrich S.L.,
 Andrews V.H., Lingner J., Harley C.B. and Cech T.R.
 TITLE Telomerase catalytic subunit homologs from fission yeast
 and human
 JOURNAL Science 277 (5328), 955-959 (1997)

30 MEDLINE 97400623
 REFERENCE 2 (residues 1 to 1132)

35 AUTHORS Meyerson M., Counter CM., Eaton EN., Ellisen LV., Steiner F.,
 Caddle SD., Ziaugra L., Beijersbergen RL., Davidoff MJ., Liu Q., Bacchetti
 S., Haber DA and Weinberg RA.
 TITLE hEST2, the putative human telomerase catalytic subunit
 gene, is up-regulated in tumor cells and during immortalization

40 JOURNAL Cell 90 (4), 785-795 (1997)
 MEDLINE 97433088
 PUBMED 9288757
 REFERENCE 3 (residues 1 to 1132)

Figure 7

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US01/05143

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7): *Phas: See Extra Sheet.
US CL : 3'Phas: See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC*

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 42/185.1, 198.1, 277.1; 51/42, 13, 14, 15, 16, 530/323, 326, 327, 328, 329

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
WEST 2.0, MEDICINE COMPENDIUM DATABASE (DIALOG), BIOTECH COMPENDIUM DATABASE (DIALOG) search terms: subunit, telomerase, hist, hist, telomerase, hist, vacuole, cancer, tumor

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 94/03205 A1 (KUBO et al.) 17 February 1994, see entire document.	1-18
Y	US 5,962,320 A (ROBINSON) 05 October 1999, see column 11.	1-18
Y	VONDERHEIDE et al. The Telomerase Catalytic Subunit Is a Widely expressed "Tumor-associated Antigen Recognized by Cytotoxic T Lymphocytes. Immunity. June 1999, Vol. 10, No. 6, pages 673-679, see entire document.	1-18
<input type="checkbox"/> Number documents are listed in the continuation of Box C.		<input type="checkbox"/> See patent family Annex.
* Special categories of cited documents:		
"A" documents defining the general state of the art which is not considered to be of particular relevance.		
"B" documents published on or after the international filing date which are not relevant to the application in question, but which may form the basis for a priority claim in another application or which may be cited to establish the priority of the present application.		
"C" documents which are not relevant to the application in question, but which may be cited to establish the priority of another application or other special reasons (as specified)		
"D" documents referring to an application, an exhibition or other sources.		
"E" documents published prior to the international filing date but later than "A" for priority date purposes.		

Date of the actual completion of the international search

12 APRIL 2001

19 MAY 2001

Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Washington, D.C. 20231 Fax: (202) 305-3230 Facsimile No. (703) 305-3230	Authorized officer RON SCHWADRON Telephone No. (703) 305-1998 *
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Form PCT/ISA/210 (second sheet) (July 1998) *

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US01/05143

A. CLASSIFICATION OF SUBJECT MATTER

IPC (7):
A61K 38/00, 38/04, 38/03, 38/08, 38/10, 39/00, C07K 9/00, 7/00, 7/04, 7/08, 7/06

A. CLASSIFICATION OF SUBJECT MATTER:

US CL :

42/185.1, 198.1, 277.1; 51/42, 13, 14, 15, 16, 530/323, 326, 327, 328, 329

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 42/185.1, 198.1, 277.1; 51/42, 13, 14, 15, 16, 530/323, 326, 327, 328, 329

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Form PCT/ISA/210 (extra sheet) (July 1998) *